Cord Blood Transplantation and the Potential for Gene Therapy

Gene Transduction Using a Recombinant Adeno-Associated Viral Vector[a]

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Hematopoietic stem and progenitor cells from different tissue sources, including adult bone marrow, growth-factor mobilized adult peripheral blood, and umbilical cord/placental blood collected at the birth of a child have been used for transplantation.[1-6] These transplants were done to repopulate the hematopoietic system of individuals whose blood cells were compromised by disease or by chemotherapy/irradiation treatment to cure a disease. Cord blood is a rich source of hematopoietic stem and progenitor cells, especially of the more immature/primitive subsets of these cells.[7-14] The first successful cord blood transplant was performed in October 1988 for a male with Fanconi anemia using HLA-matched cells from his sister.[15] Now more than 6 years have passed since the transplant. The recipient's blood system is essentially completely repopulated with his sister's cells and he is cured of the hematologic manifestations of Fanconi anemia. Since this initial transplantation performed on the basis of a prior biological assessment of the proliferation of stem/progenitor cells in cord blood,[7] over 60 cord blood transplantations have been per-

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formed using complete HLA-matched or, less frequently, 1-, 2-, or 3-HLA antigen disparate sibling cord blood cells.[15-30] This information was obtained through a newly established international cord blood transplant registry.[17,29,31] Transplantations have been done for: Fanconi anemia, aplastic anemia, β-thalassemia, severe combined immunodeficiency, x-linked lymphoproliferative disease, Hurler's syndrome, Hunter's syndrome, Wiskott-Aldrich Syndrome, acute and chronic myelogenous leukemia, acute lymphocytic leukemia, juvenile chronic myelogenous leukemia, myelodysplasia, and neuroblastoma. Of the first 50 evaluated transplants in which HLA-matched or 1-antigen mismatched cord blood was used, the survival of recipients after 1.5 years was about 70%, of which 54% was disease-free survival.[17] Since the initial reports of successful cord blood transplants, banks have been established to store cord blood frozen in cryopreserved form for potential use in autologous and allogeneic transplantation.[32-36] Presently, more than 35 frozen cord blood specimens from such banks, which were unrelated to the recipient and either HLA-matched or 1- to 3-HLA-antigen mismatched, have been used for transplantation. Thirty-five of these unrelated samples came from the cord blood bank directed by Dr. Pablo Rubinstein at the New York Blood Center. Most recipients of unrelated cord blood engrafted. No published reports of these unrelated cord blood transplants currently exist. All related and most unrelated transplants were performed in children. The weight of the heaviest recipient thus far transplanted and engrafted with cord blood is about 70 kg. One 40-kg child with acute lymphoblastic leukemia underwent transplantation at the Indiana University School of Medicine directed by Drs. David Emanuel and Franklin Smith with unrelated cord blood cells from the New York Blood Center's cord blood bank. Of interest and clinical relevance is the limited graft-versus-host disease noted in related and unrelated cord blood transplants. This may reflect the low immune reactivity of cord blood T lymphocytes[37-39] and natural killer cells.[40]

Primitive progenitors in cord blood have phenotypically been characterized as being $CD34^{3+}$,[11] $CD34^{+}CD45RA^{lo}CD71^{lo}$,[12] $CD34^{+}CD38^{-}$,[13] $CD34^{+}thy1^{+}$,[41] and $CD34^{+}HLA\text{-}DR^{+}$.[42,43] Functionally these cells are slowly cycling,[42-45] but very sensitive when stimulated to proliferate.[7-14] They have high proliferative and replating capacity[7-14,46] and can be greatly expanded *ex vivo*.[8,10,12,13,42,43,45,47-52] Stem/progenitor cells from human cord blood also can extensively engraft the marrow of sublethally irradiated mice with severe combined immunodeficiency disease.[53,54]

Many questions remain regarding umbilical cord blood hematopoietic stem and progenitor cells and their use in transplantation. These questions, which have been discussed elsewhere,[55] include: the use of cord blood for transplantation of adults, the potential uniqueness of stem and progenitor cells in cord blood, the possibility of *ex vivo* expansion of long-term marrow repopulating cells in cord blood, the immunologic reactivity of cord blood cells, the separation of cord blood stem and progenitor cells for use in transplantation, the banking of cord blood cells in a cryopreserved form, and the use of these cells for gene transduction and possible gene therapy to treat genetic or other disorders. This latter possibility forms the basis for our experiments with the use of adeno-associated viral (AAV) vectors to place new genetic material into stem/progenitor cells from human cord blood.

Both retroviral[56-61] and AAV[62,63] vectors have been used to place genes into more mature subsets of stem cells and immature and mature subsets of progenitor cells

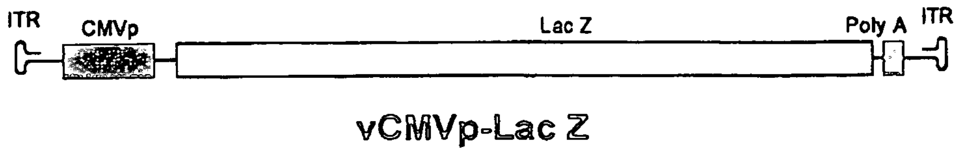

FIGURE 1. Schematic representation of recombinant adeno-associated viral vector containing the *lacZ* gene under the control of a cytomegalovirus (CMV) promoter (p). The *lacZ* gene contained a nuclear localization factor. ITR = inverted terminal repeat.

from cord blood. The present study involves the use of a recombinant AAV vector containing the CMV promoter-driven β-galactasidase (*lacZ*) gene to demonstrate efficient transduction and expression of the β-galactasidase gene in immature subsets of myeloid progenitor cells from cord blood.

MATERIALS AND METHODS

Cells and Cell Separation. Cells were obtained from normal human umbilical cord blood scheduled to be discarded after delivery of the infant and after the prior need for samples for clinical study had been satisfied. Low-density cells were obtained after density cut using Ficoll-Hypaque (density, 1.077 g/cm^3; Pharmacia Piscataway, New Jersey. CD34^{3+} cells were obtained after sorting nonadherent low-density T-lymphocyte-depleted cells on a FACS Star Plus Cell Sorter (Becton-Dickinson, San Jose, California.)[57] This population was >98% pure for cells expressing the CD34 antigen. CD3^{3+} cells included 20% of CD34 antigen-expressing cells with highest density distribution of CD34 antigens. This fraction is richest in stem/progenitor cells.[11]

Vector and Transduction Procedure. A diagram of the recombinant AAV vector used, vCMVp-LacZ, is shown in FIGURE 1. This vector was prepared in a manner similar to that used for a vector containing the neo-phosphotransferase sequence,[62] except that expression of LacZ was driven by the cytomegalovirus (CMV) promoter. Freshly isolated low-density or CD34^{3+} cord blood cells were either mock-infected or infected with vCMVp-LacZ virions at a multiplicity-of-infection of 1 as described elsewhere for other viral vectors before washing the cells twice and plating them in semisolid culture medium for assessment of progenitor cells.[62]

Colony Assays and Detection of β-gal$^+$ Cells. Low-density (2.5×10^4/ml) or CD34^{3+} (200/ml) cells were plated in 1% methylcellulose culture medium with 30% fetal bovine serum (Hyclone, Logan, Utah) in the presence of 1 U/ml recombinant human (rhu) erythropoietin (Epo; purchased from Amgen, Thousand Oaks, California), 100 U/ml rhu granulocyte-macrophage colony-stimulating factor (GM-CSF), 100 U/ml rhu interleukin (IL)-3, and 50 ng/ml rhu steel factor (SLF) for assessment of immature subsets of granulocyte-macrophage (CFU-GM) and multipotential (CFU-GEMM) progenitor cells and in the presence of these concentrations of Epo, GM-CSF, and IL-3 for assessment of more mature subsets of CFU-GM and BFU-E.[8] GM-CSF, IL-3, and SLF were kind gifts from Immunex Corp, Seattle, Washington.

TABLE 1. Transduction of Immature Subsets of Cord Blood Myeloid Progenitor Cells with a Recombinant Adeno-Associated Viral (AAV)-LacZ Vector[a]

Cells	β-Gal$^+$ Colonies (%)	
	CFU-GM	CFU-GEMN
Mock-infected	5 ± 2 (1-9)[b]	10 ± 2 (4-14)
vCMVp-LacZ-infected	28 ± 12[c] (7-59)	53 ± 5[c] (45-67)

[a] Low-density or CD34^{3+} cord blood cells were either mock infected or infected with the recombinant AAV-LacZ virions. Cells were treated and plated as described in Materials and Methods. Colonies containing β-galactosidase-expressing cells were assessed after 14 days of incubation for cells from four separate experiments (two using low-density cells and two using CD34^{3+} cells with over 1,000 colonies evaluated). Although the *lacZ* gene contained a nuclear localization factor, no attempts were made to distinguish nuclear from cytoplasmic staining in these experiments. Results are expressed as mean ± 1 SEM.

[b] Range of percentages of β-gal$^+$ colonies.

[c] Significant difference from mock-treated cells by Student's *t* test, $p < 0.001$.

We previously demonstrated[8] and it has been substantiated[10] that in the presence of SLF (also called stem cell factor) with other colony-stimulating factors, CFU-GEMM but not BFU-E colonies can be detected in cord blood. Thus, results are shown for CFU-GM and CFU-GEMM when cord blood is stimulated with Epo, GM-CSF, IL-3, and SLF. Colonies were scored after 14 days of incubation in 5% CO_2 and lowered (5%) O_2. Twenty-four hours before scoring the colonies, a 0.1-ml solution containing 150 µg X-galactoside was added to all 1-ml plates.

RESULTS AND DISCUSSION

To evaluate the expression of new genetic material transduced into myeloid progenitor cells from cord blood, we used a recombinant AAV vector containing the *LacZ* gene under the control of the CMV promoter. As shown in TABLE 1, we detected expression of β-galactosidase in the progeny (cells within colonies) of immature subsets of CFU-GM and CFU-GEMM incubated with the vCMVp-LacZ AAV vector and then stimulated to proliferate in semisolid culture medium in the presence of the combination of Epo, GM-CSF, IL-3, and SLF. The results shown are averages from four separate experiments in which either low-density ($n = 2$) or CD34^{3+} ($n = 2$) cells were transduced. Some background staining of colonies derived from mock-treated cells was detected, but the percentage of β-gal$^+$ colonies deriving from the lacZ-containing vector-infected cells was significantly higher and many times greater than that of the mock-infected cells. It is not clear what caused the background staining, but this may represent endogenous expression of proteins that can act on the X-gal substrate. The background level of β-gal$^+$ colonies varied from 1-14%, making it clear that the true expression potential of such transduced cells cannot be assessed

without an appropriate control. Examples of positive transduced and negative control colonies are shown in FIGURE 2a-d. As seen in FIGURE 2e-h, expression of β-gal was also seen in colonies deriving from more mature subsets of CFU-GM and BFU-E transduced with the *lacZ* gene and stimulated to form colonies with Epo, GM-CSF, and IL-3. Variability was noted between colonies in the percentage of β-gal$^+$ cells per colony. It is currently not clear if gene expression occurs in only a portion of transduced cells. Actual transduction efficiency determination awaits analysis by polymerase chain reaction amplification of the DNA of the transduced gene from colony cells.

The use of recombinant AAV vectors to efficiently transduce cord blood stem/progenitors with genes does not appear to require preincubation of the cells with growth factors[62] as is necessary for high efficiency transduction of these cells with retroviral vectors.[59] Because preincubation of stem/progenitor cells with growth factors could possibly cause differentiation of the earliest cells, those with long-term marrow-repopulating ability that would be desirable to transduce, the use of AAV vectors in some circumstances may be more advantageous than that of retroviral vectors. The lack of the need for growth factor preincubation for efficient transduction with AAV vectors of slow or noncycling cells, such as those found in cord blood,[40–45] does not mean that cell division is not necessary for integration of the gene. Integration of new genetic material may still require cell division which can occur in response to growth factors after the preincubation phase in which cells are exposed to viral vectors.

For gene transduction with recombinant AAV vectors to be of use in gene therapy, it is important to know if expression of the introduced gene is still high after expansion of the transduced cell population *in vivo* or *ex vivo*. In this context, we expanded immature subsets of cord blood CFU-GM and CFU-GEMM, after 7 days in suspension culture with a combination of growth factors, by greater than 60- and 6-fold, respectively, with maintenance of high level expression of the *lacZ* gene introduced by the recombinant AAV-LacZ vector (unpublished data). Although more experimentation is needed in this area, the results suggest that long-term expression of genes introduced by recombinant AAV vectors may be possible. Unfortunately, without a quantitative assay for human stem cells with long-term marrow-repopulating capacity,[64] we do not yet know the transduction efficiency of this cell type and whether this cell is expanded, maintained, or lost after efforts at *ex vivo* expansion of these rare but important cells.

In the future it may be possible to enhance *ex vivo* expansion of stem and progenitor cells by placing genes into these cells for cytokines or receptors for cytokines. Moreover, recent preliminary studies using retroviral vectors suggest that stem/progenitor cells purified from cryopreserved cord blood could be transduced with high efficiency and expanded *ex vivo* with stable integration and expression of the introduced gene.[65] Also, transduced cells could be frozen and recovered with the thawed cells expressing the introduced gene.[66] The latter reports[65,66] suggest that it may be possible to alter stem and progenitor cells by introducing new genetic material either before or after banking these cells in a cryopreserved state.

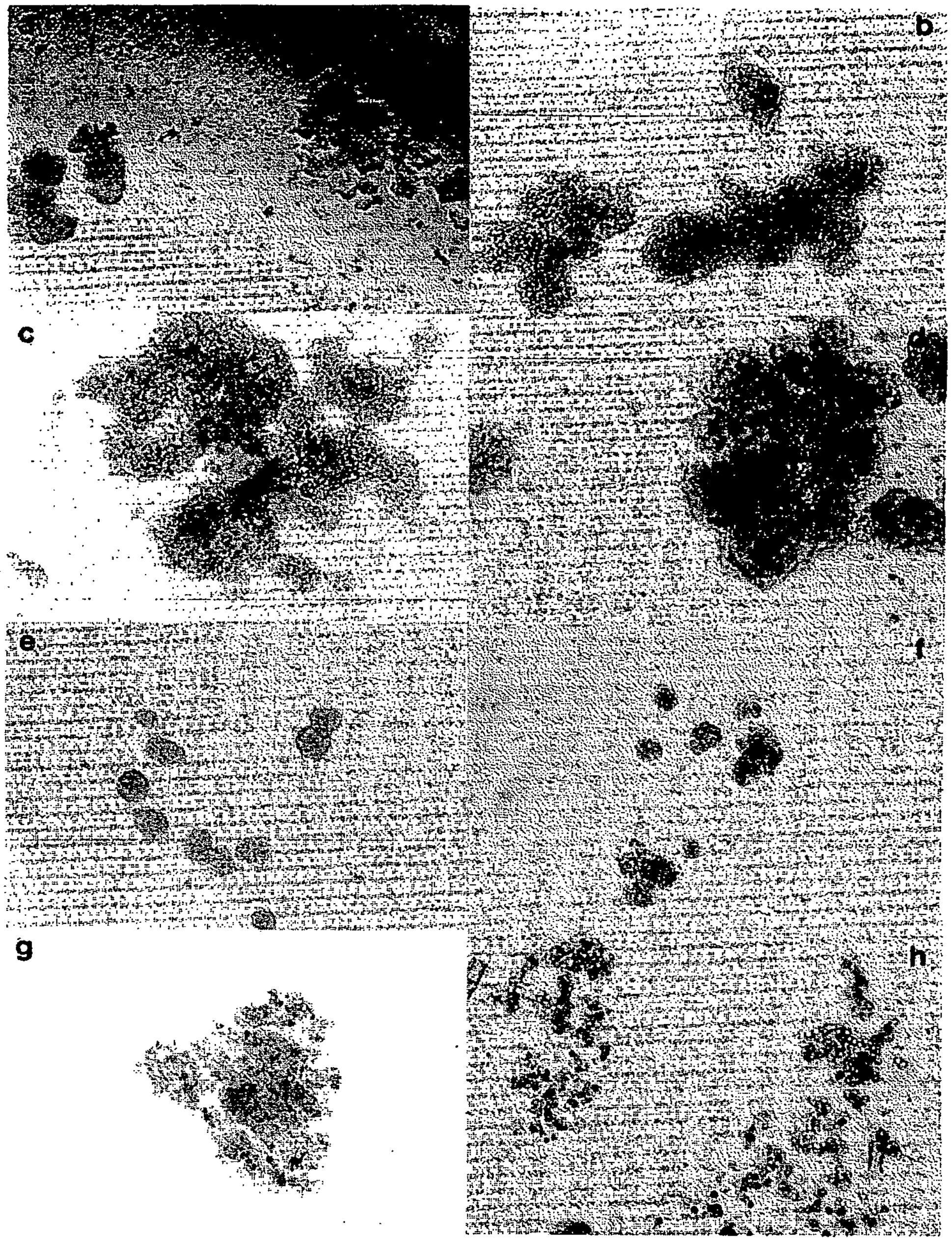

FIGURE 2. Examples of expression of the β-galactosidase gene in cells of colonies derived from multipotential (CFU-GEMM) (**b–d**) and erythroid (BFU-E) (**f** and **g**) and granulocyte-macrophage (CFU-GM) (**h**) progenitor cells from $CD34^{3+}$ cord blood cells transduced with the recombinant adeno-associated viral (AAV) LacZ vector. Magnification × 40. The CFU-GEMM colonies were photographed after 14 days of incubation of cells grown in the presence of Epo, IL-3, GM-CSF, and SLF, whereas BFU-E and CFU-GM colonies were photographed after growth of cells in the presence of Epo, IL-3, and GM-CSF. Panels **a** and **e**, respectively, show CFU-GEMM and BFU-E colonies derived from mock-infected cells, which did not stain.

SUMMARY

Cord blood, which contains a high frequency of immature stem/progenitor cells with extensive proliferative and replating capacity *in vitro* was used as a clinical source of transplantable stem and progenitor cells. These cells can be efficiently transduced with new genetic material by using AAV or retroviral vectors. Using a recombinant AAV vector, high level expression of the *lacZ* gene under a CMV promoter was demonstrated in immature subsets of cord blood progenitor cells.

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